

# Cell boundaries: knowing who to mix with and what to shout or whisper

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The importance of cell position in relation to boundaries in development goes back to Hans Driesch and his experiments over 100 years ago in the sea urchin embryo. These experiments convinced him that the way a cell developed depended on its position in the embryo. Driesch believed that the embryo had a system of coordinates, like  $x$  and  $y$  axes, that specified the position of cells within the embryo in order to determine their behaviour. He was, however, equally convinced that it was not physically possible to have such a system and invoked a mystical concept, which he called 'entelechy', to explain the positioning of cells during development. The first evidence that cells do indeed receive position-specific information came from E. N. Browne, who showed in 1909 that the hypostome, the mouth region of the adult hydra, could induce a whole new axis when grafted into the body of another hydra. But the key experiment to prove that cells can signal and pattern adjacent cells came in 1924 when Hans Spemann and Hilde Mangold demonstrated that a specific region of the frog embryo, the organiser, could specify a whole new axis.

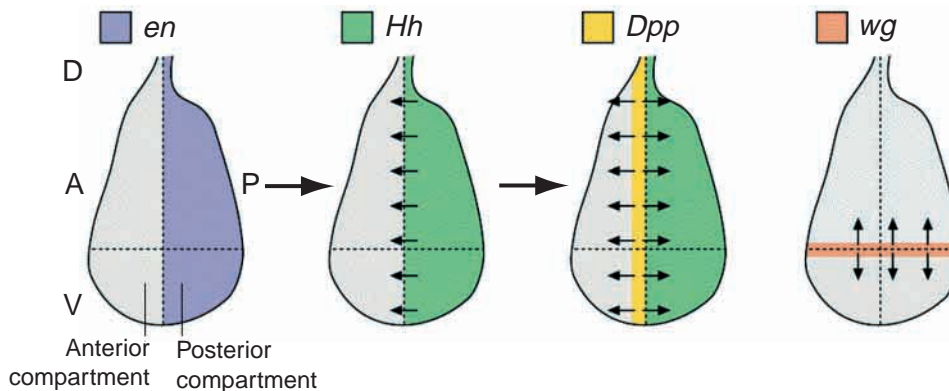
The aim of the meeting 'Boundaries in development: 30 years of progress', which took place at the EMBL in Heidelberg in June this year, was to understand the role of specific boundary regions, in both *Drosophila* and vertebrates, that keep populations of cells separate and act as signalling regions that pattern the cells on both sides of a boundary. But why are there boundaries in the developing embryo? The reason relates to patterning and size. The signals that are involved in specifying the spatial pattern of cell behaviour in the embryo act over rather short distances, rarely more than about 30 cell diameters. Thus, as the embryo grows bigger, patterning becomes a problem. Evolution found the solution by

separating the whole embryo into semi-autonomous units, the development of which is almost entirely independent from adjacent regions. This requires that boundaries develop to separate these regions and also to act as signalling centres for the regions adjacent to them.

The modern interest in boundaries and how they might specify the position of a cell dates back to the work on insect segments, as Peter Lawrence (Laboratory of Molecular Biology, Cambridge, UK) made clear (Casal et al., 2002). In 1973, Lawrence observed that a gap in the boundary between two segments of the insect *Oncopeltus* results in a local reversal of polarity (as shown by the direction of the hairs on the segment). This observation highlighted the importance of this boundary for establishing positional information. Lawrence suggested from this work that, if the gradient of a morphogen within a segment specified the polarity of the segment, a gap in the boundary might permit the morphogen to flow back into the adjacent segment, thus reversing the polarity of the hairs (Lawrence, 1992). Another early discovery was recounted by Antonio Garcia-Bellido (Universidad Autonoma, Madrid, Spain), who, 30 years ago, reported the existence of cell compartments in the *Drosophila* wing imaginal disc, which are defined by boundaries between them (Garcia-Bellido et al. 1973). He showed that there was a lineage restriction across a boundary that divided the wing disc into anterior and posterior compartments (see Fig. 1). The posterior compartment was specified by the engrailed (*en*) gene, which was thought to define cell affinities and so prevent them from mixing with cells in the anterior compartment. Garcia-Bellido also introduced the concept of a selector gene – a gene that determines the properties of a group of cells. His early work showed that boundaries not only separate cells that have different gene expression patterns, but also act as signalling centres. So, the key questions that these early studies raised are: how are boundaries specified and maintained, and what is the nature of the signal they produce?

## Maintaining boundaries in the fly

Today, we still do not understand just how that beautifully straight anterior-posterior (AP) boundary that exists in the fly wing imaginal disc is maintained. Although Garcia-Bellido has shown that anterior and posterior disc cells will, when mixed in a culture, sort into anterior and posterior cell groups, there is still no evidence that it is cell affinities that establish the



**Fig. 1.** The establishment of signalling regions at compartment boundaries in the *Drosophila* wing disc. The gene engrailed (*en*) is expressed in the posterior compartment of the wing disc together with hedgehog (*Hh*). At the boundary with anterior cells, *Hh* activates Decapentaplegic (*Dpp*), which is secreted into both compartments. At the dorsal-ventral (DV) boundary, wingless (*wg*) is expressed. Reproduced, with permission, from Wolpert et al. (Wolpert et al., 2002).

compartment boundaries, nor has the nature of the cell-surface molecules responsible been identified. However, there is evidence that certain transmembrane proteins play a role in boundary maintenance (Milan et al., 2001). Seth Blair (University of Wisconsin, Madison, WI, USA) discussed how compartment boundaries are maintained by somewhat different mechanisms in the notal and hinge regions of the wing disc, as cells can cross the notal-hinge boundary. Moreover, Ken Irvine's (Rutgers University, New York, NY, USA) studies on the dorsal-ventral boundary in the wing disc provides evidence for a very different mechanism to that based only on cell affinities. He reported that a stripe of Notch activation is able to act as a type of a fence that keeps the cells of the dorsal and ventral compartments apart. The cytoskeleton appears to be involved in the establishment of this fence, as cells at this boundary are elongated and express increased levels of F actin; in addition, profilin, which interacts with actin, can disrupt this boundary.

In the early *Drosophila* embryo, boundaries also exist between parasegments and segments, which are specified by a mechanism that involves Hedgehog (Hh), Wingless (Wg) and En. Jean-Paul Vincent (NIMR, London, UK) has looked at cell shape changes that accompany the formation of these boundaries, and has found that *en*-expressing cells undergo apical constriction and adopt a bottle shape, studies that again implicate the cytoskeleton as being involved in the determination of cell shape at a boundary. Christian Dahmann (Max Planck Institute of Molecular Cell Biology, Dresden, Germany) also discussed how Decapentaplegic (Dpp), a TGF $\beta$ -receptor ligand, might contribute to maintaining boundaries in the wing disc. Dpp is activated in a row of anterior cells, some eight to ten cells wide, in the anterior compartment by Hh that is expressed from the posterior compartment. Cells that cannot respond to Hh move to the

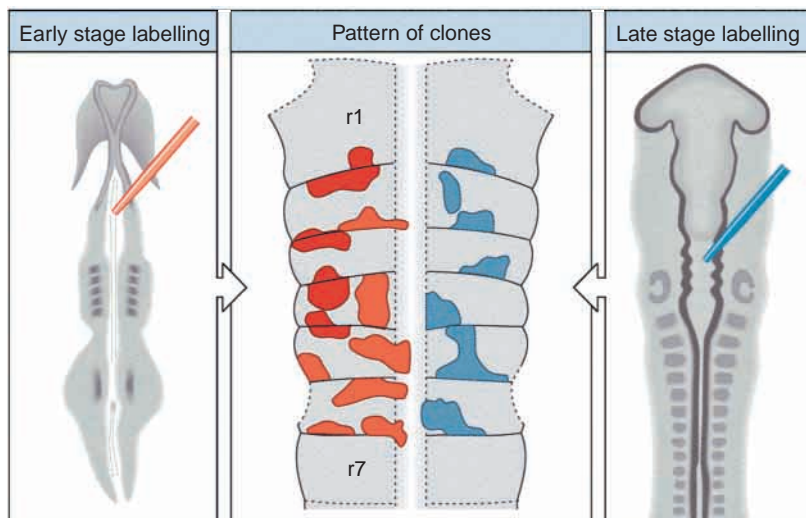
posterior of the boundary, indicating that Dpp expression is required to help maintain the boundary, as only anterior cells respond to Hh by turning on Dpp expression.

### Boundaries in vertebrates

In vertebrates, the mechanisms of boundary formation are even less well understood. Segmentation in the hindbrain plays a fundamental role in the formation of the CNS, as this region of the brain is divided up into rhombomeres, the boundaries of which are lineage restricted (see Fig. 2). During embryogenesis, neural crest cells from the rhombomeres move into different branchial arches; the migration pattern of these cells is determined by their local environment. As Robb Krumlauf (Stowers Institute for Medical Research, Kansas City, MO, USA) has shown, the activity of the Slit and Robo signalling pathways, which were originally identified as controllers of neurone migration across the midline of the fly, play a key role in directing this migration (Trainor et al., 2002). But how the boundaries are initially specified is not known, although David Wilkinson (NIMR, London, UK) has good evidence that bi-directional signalling by activation of the Eph receptors by Ephrin B proteins restricts the intermingling of cells across the rhombomere boundary (Xu et al., 1999).

Similarly, little is known about the specification of the boundary that separates the midbrain and hindbrain, which also acts as a signalling centre during vertebrate embryogenesis. Evidence presented by Michael Brand (Max Planck Institute of Molecular Cell Biology, Dresden, Germany) showed that repressive interactions between the homeodomain transcription factors Otx and Gbx contribute to the positioning of this boundary, and that Wnt8 is involved in its initial positioning (Rhinn and Brand, 1999). It remains unclear whether this boundary is one of lineage restriction. Four Fgf proteins, as well as Wnt1, are expressed at this boundary, but as Alex Joyner (Skirball Institute, New York, NY, USA) pointed out, only Fgf8 seems to have organiser activity here: it can induce cerebellum, while the other Fgf proteins can induce only expansion of the midbrain (Wassef and Joyner, 1997). With regards to the brain itself, Eddy De Robertis (University of California, San Francisco, CA, USA) provided evidence in *Xenopus* of a novel signalling centre in the blastula, the pre-organiser, which is required for neural induction (Oelgeschlager et al., 2003). This organising region can account for experiments that have provided evidence for the existence of a planar signal. This signal arises from within the neural tissue itself, and is distinct from the vertical signals that arise from the underlying mesoderm and pattern the overlying neural tissue.

Another boundary in the vertebrate brain, which was described at the meeting by Clemens Kiecker (MRC Centre for Developmental Neurobiology, Kings College, London, UK), is the zona limitans intrathalamica, which is present in the chick forebrain and might also have a signalling function through the action of sonic hedgehog (Shh). This boundary arises from a wedge-shaped region in the prosencephalon, and both its anterior and posterior borders are lines



**Fig. 2.** Lineage restriction in the rhombomeres of the embryonic chick. Single neuroepithelial cells labelled at early (during somite formation, left, red and orange) and at later (right, blue) stages of development are shown. The middle panel shows their labelled descendants mapped 2 days later. Cells labelled early and before boundaries have formed have descendants in more than one rhombomere (r). Reproduced, with permission, from Wolpert et al. (Wolpert et al., 2002) [adapted from Lumsden (Lumsden, 1991)].

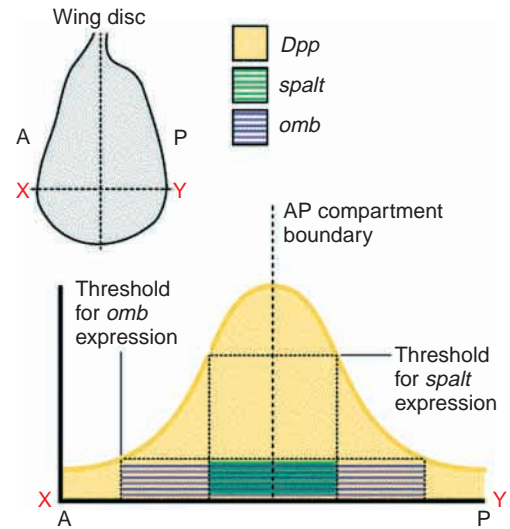
of lineage restriction (Larsen et al., 2001). This boundary is clearly a special region as it does not express *lunatic fringe* (*Lfng*), a gene that is involved in somite boundary formation, although *Lfng* is expressed in the cells that are present on either sides of the boundary. Patrick Charnay (INSERM, Paris, France) reported on a different type of boundary in the chick nervous system. At this boundary, special cells of neural crest origin condense where motoneuron axons leave the spinal cord. These cells prevent the axon cell body from migrating with the axon, and so from exiting from the spinal cord (Vermeren et al., 2003).

Notch pathway activation was invoked in many of the systems discussed at this meeting, and it clearly plays a role in the formation of the repeated boundaries that result from somite formation. David Ish-Horowicz (Cancer Research UK, London, UK) reported on the role of Notch in the oscillator, which results in the oscillation of *Hairy 1* in the chick node during its regression, as well as in the presomitic mesoderm. This oscillator appears to control somite segmentation, and Ish-Horowicz emphasised the role of cycling *Lfng* in maintaining the oscillation, while Notch coordinates cell behaviour (Pourquié, 2002). Olivier Pourquié (Stowers Institute for Medical Research, Kansas City, MO, USA), who originally identified the oscillation of *Hairy1* in the presomitic mesoderm, discussed the key role of *Fgf8* in somite segmentation. *Fgf8* is expressed in a gradient in the presomitic mesoderm, being at its highest posteriorly in the node region. Only when cellular *Fgf8* reaches a sufficiently low level do cells become determined and form somites (Dubrulle and Pourquié, 2002). The concentration of *Fgf8* thus forms a type of threshold boundary. Surprisingly, Paul Kulesa (Stowers Institute for Medical Research, Kansas City, MO, USA) has found, by filming chick somite formation, that cells move across presumptive somite boundaries and do not appear to be assigned to a given somite when they leave the node (Kulesa and Fraser, 2002). A rather different boundary of sorts is that relating to the establishment of left-right asymmetry by Notch activation. Juan Carlos Izpisua-Belmonte (Salk Institute, La Jolla, CA, USA) discussed the role of Notch in this process and, using many equations, invoked a  $H^+$  transport mechanism that might specify left-right determination, together with *Lfng*. In this model, Notch activity may be triggered by a primary gradient in  $H^+/K^+$ -ATPase activity that specifies the left-right axes (Levin et al., 2002).

### Boundaries and specification of positional values

One model for patterning tissues is that cells acquire a positional identity with respect to the boundaries nearest to them; this identity is then interpreted by cells in terms of their developmental history. Thus, the same set of positional values can be used to generate very different patterns, as in insect imaginal discs. Several models suggest that the way position is specified is by a graded signal, which is established from a boundary or organiser. Such models raise several key questions: what the nature of such signals might be, how such signals might be interpreted by a cell and how fine-grained the positional values are that they create. Does every cell in the wing disc, for example, have a unique positional value with respect to all the other cells in the disc?

One of the best-studied systems for the specification of positional values by a morphogen gradient is the Dpp signal from the AP boundary of the fly wing imaginal disc, which



**Fig. 3.** Patterning of the wing disc along the anterior-posterior (AP) axis. Decapentaplegic (Dpp) protein is assumed to be a diffusible morphogen that forms an asymmetric gradient in both compartments. *spalt* and *omb*, targets of Dpp that are activated at specific threshold concentrations of Dpp. Reproduced, with permission, from Wolpert et al. (Wolpert et al., 2002).

Steven Cohen (EMBL, Heidelberg, Germany) has studied intensively (Teleman and Cohen, 2000). His view is that Dpp diffuses into both compartments of the wing disc and sets up a basic pattern by, for example, activating *spalt* (see Fig. 3) in a region that is determined by threshold concentrations of Dpp. Dpp also has a major role in establishing the wing-notum boundary, which Juan Modolell (Centro Biología Molecular Severo Ochoa, Madrid, Spain) has shown is not one of lineage restriction; yet the cells here give rise to different structures on either side of the boundary. Homeobox genes of the Iroquois complex are expressed in the notum, and when they are not present, the cells there become wing hinge cells.

Personally, I find it very hard to understand how the diffusion of a morphogen could, on its own, reliably specify fine-grained positional values, as this model does not take into account how the binding of a morphogen to its receptors, and receptor saturation, might affect the distribution of a morphogen. For example, a gradient in receptor activation may not occur as a result of the simple diffusion of a morphogen, as receptors will become saturated (Kerszberg and Wolpert, 1998), particularly given that time-lapse films of GFP-labelled morphogens that were shown at the meeting appear to me to show that morphogens behave almost chaotically. The same is true of models that are based on the transport of morphogens from the boundary in vesicles called argosomes, as proposed by Suzanne Eaton (Max Planck Institute of Molecular Cell Biology, Dresden, Germany) (Greco et al., 2001). Such vesicles contain heparin sulphate proteoglycans that can modify the activity of morphogens such as Dpp, Hh and Wnt proteins. By contrast, Thomas Kornberg (University of California, San Francisco, CA, USA) has investigated a mechanism in which long cell-extensions in the wing disc, similar to filopodia and called cytonemes, might play a role in specifying position with respect to the AP wing boundary, which they extend towards (Ramirez-Weber and Kornberg, 1999). But, again, there is no plausible

model for how they could be involved in specifying position with respect to that boundary.

Garcia-Bellido referred to the old regeneration experiments of Horst Bohn, who cut out pieces of the cockroach leg to produce the regeneration of the missing region, and thus the precise intercalation of the missing positional values. This is similar to the intercalation of positional values when segments of the wing disc are removed. Garcia-Bellido suggested that a similar process may be involved when the disc grows; once boundaries are established, a cell at the boundary could intercalate further positional values and could thus specify a fine-grained pattern of positional values on a cell-by-cell basis. However, the mechanism underlying such fine-grained patterning, if it does involve intercalation, is totally unknown (Resino et al., 2002). There is also evidence against intercalation occurring in the fly wing disc, as activation of the Dpp receptor locally should lead to a positional value similar to that at the boundary; therefore, intercalation should result in a large number of new values. This does not occur unless a high level of Dpp is specified locally (Nellen et al., 1996). But, as Peter Lawrence has suggested, cell specification in the fly wing disc might occur level by level, as it does in the fly embryo, as reflected by the increasingly restricted expression patterns of certain genes, such as *omb* (*bi* – FlyBase) and *spalt*. One hypothesis of my own is that a diffusible morphogen might set up the initial polarity of a tissue and then another mechanism, possibly based on the propagation of cell polarity, then specifies positional identity (Ma et al., 2003).

### Unanswered questions

Although there has been significant progress in understanding how boundaries form and signal over the past 30 years, many key questions regarding their establishment and function still remain unanswered. These include how boundaries, particularly in the vertebrate brain, are specified (an important question because they underlie the patterning of the brain), how the position of a cell is specified with respect to boundaries and how position is recorded by a cell. It is particularly striking that in the newt, a cell-surface protein, Prod 1, is expressed as a continuous gradient along the limb (da Silva et al., 2002) and that retinoic acid can alter positional identity in the regenerating limb of this organism in a graded manner (Maden, 1996). It would be surprising if the mechanisms involved in these events were not widely used. Indeed, there are some striking similarities in the mechanisms of cell patterning between insects and vertebrates, and similar signalling molecules, such as Fgf proteins and Hh, are used again and again. If cells use gradients, they will have reliable mechanisms for setting them up. However clever one thinks cells in the embryo are, they will turn out to be cleverer. This excellent meeting made us all think hard about these questions, and about the experimental tools and expertise that we will need to tackle them.

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